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Original Research Article

Salinity Alters the Polyisoprenoid Alcohol Content and Composition of Both Salt-Secreting and Non–Salt-Secreting Mangrove Seedlings

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ABSTRACT

The effects of salinity on the polyisoprenoid alcohol content and composition of the salt-secreting mangrove species *Avicennia marina* and *Sonneratia alba* and the non–salt-secreting species *Bruguiera gymnorrhiza* and *Kandelia obovata* were studied. The seedlings of mangroves were grown for 5 months under 0% and 3% salt concentrations. The occurrence, content, and distribution of four mangrove seedlings were analyzed by two-dimensional thin layer chromatography. The structural groups of the polyprenols and dolichols in the leaves and roots were classified into two types (I and II). In type I, dolichols predominated over polyprenols (more than 90%), whereas in type II, the occurrence of both polyprenols and dolichols was observed. Polyprenols were not detected in the leaves of *A. marina* and *B. gymnorrhiza* under 0% salt (control), but were detected in small amounts in *K. obovata* leaves; however, significant amounts were found in the 3% salinity group. This finding in *A. marina*, *B. gymnorrhiza*, and *K. obovata* leaves implies a change to the structural group: under 0% salt concentrations, the groups are classified as type I, but become type II under 3% salt concentrations. The occurrence of ficaprenol (C_{50-55}) was found only in the leaves of the non–salt-secreting species *B. gymnorrhiza* and *K. obovata* under 3% salinity and not in the salt-secreting species *A. marina* or *S. alba*. It is noteworthy that the polyisoprenoid type in the roots of the four species showed no change under salinity; the two salt-secreting species *A. marina* and *S. alba* contained type I under 0% and 3% salt concentrations. On the other hand, type II polyisoprenoids were identified in the non–salt-secreting species *B. gymnorrhiza* and *K. obovata* under 0% and 3% salinity conditions. This finding suggested that polyisoprenoids play a protective role against salinity in the mangrove leaves of both salt-secreting and non–salt-secreting species.

1. Introduction

Mangrove plants are distributed in the intertidal zone of tropical and subtropical regions and are defined physiologically by their ability to grow under various levels of salinity, ranging from freshwater to hypersaline environments (Tomlinson 1986). Mangrove plants fall into three groups based on their approach to managing salinity tolerance: salt secretors, non–salt-secretors, and salt accumulators (Basyuni *et al.* 2012a; Tomlinson 1986). The species of salt secretors include *Avicennia marina* (Acanthaceae) and *Sonneratia alba* (Sonneratiaceae), which have either salt glands or salt hairs to remove excess salt. In contrast, non–salt-secretors, exemplified by *Bruguiera gymnorrhiza* and *Kandelia obovata* (Rhizophoraceae), do not have such morphological features for the excretion of excess salt. Salt accumulators, such as *A. marina*, *B. gymnorrhiza*, and *S. alba*, can cope with high salt concentrations in their cells (Basyuni *et al.* 2012a). *A. marina*, *B. gymnorrhiza*, *K. obovata,* and *S. alba* are common mangrove species on Okinawa Island, Japan, and are considered representatives of each group in terms of salt management strategies.

Mangroves are well known to produce secondary metabolites, including polyisoprenoids whose physiological roles remain

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unclear (Basyuni *et al.* 2016, 2017a; Skoczylas *et al.* 1994). There are two main types of polyisoprenoid alcohols in plants: polyprenols and dolichols (Figure 1). It is believed that polyisoprenoids may play a significant role in the adaptation of plants in response to adverse external stresses. The concentration of polyisoprenoids has been reported to change on biotic and abiotic stresses (Baczewska *et al.* 2014; Bajda *et al.* 2009; Zhang *et al.* 2008). Our previous studies have also demonstrated that levels of triterpenoids and triterpenoid synthase gene expression in salt-secreting and non-salt-secreting mangrove roots and leaves increase with increasing levels of salt (Basyuni *et al.* 2009, 2011, 2012a). This salt-dependent change in triterpenoid content is reversible on transfer to fresh water (Basyuni *et al.* 2012b). Aside from these metabolic shifts to overcome environmental stresses, the present study aimed to describe the effects of salinity on the polyisoprenoid alcohol content and composition of the salt-secreting mangroves *A. marina* and *S. alba* in comparison with that of the non-salt-secreting species *B. gymnorrhiza* and *K. obovata*.

2. Materials and Methods

2.1. Chemicals

A mixture of dolichol (C_{90}–C_{105}) and polyprenol (C_{90}–C_{100}) standard compounds was used to identify the polyisoprenoids that were detected in this study, as previously described (Basyuni *et al.* 2016). Silica gel 60 thin layer chromatography (TLC) glass plates and reversed-phase silica RP-18 high-performance thin-layer chromatography (HPTLC) glass plates were purchased from Merck (Darmstadt, Germany). All other chemicals and solvents were of reagent grade and obtained from Merck. The identification of the family corresponding to polyprenols or dolichols was performed for at least three experiments. The bombiprenone family (Figure 1), as described previously (Basyuni *et al.* 2016), was purified using silica gel chromatography of nonsaponifiable lipids (NSLs) from the $CHCl_3/CH_3OH$ (2:1) extracts of dry *Perilla* leaves. The purified fractions were confirmed by ESI-MS (Bruker Daltonics solariX, Manning Park Billerica, MA, USA) to have an m/z value $[M+Na]^+$ of 625.53183, which corresponded to $C_{43}H_{70}O$ (bombiprenone).

2.2. Plant material

Viviparous mature and healthy propagules (seeds) of two non-salt-secreting mangrove species, *B. gymnorrhiza* (L.) Lam. and *K. obovata* (S., L) Yong (formerly as *Kandelia candel*) (Rhizophoraceae), as well as crypto-viviparous mature and healthy seeds of two salt-secreting species, *A. marina* (Forssk.) Vierh (Acanthaceae) and *S. alba* J. Sm. (Sonneratiaceae), were collected from Iriomote Island, Okinawa, Japan, and planted in Wagner pots under varying salinity levels in a glass house. The characteristic indicators of *B. gymnorrhiza* propagule maturity were reddish-brown or greenish-red hypocotyls, a 1.7–2.0 cm diameter, and a 20–30 cm length; mature *K. obovata* propagules had yellowish-green hypocotyls and greenish-yellow cotyledons and were 20–30 cm long and 1.5–2.0 cm wide in diameter. Mature seeds of *A. marina* were green in color (similar to the pericarp color), 1.5–2.5 cm long and 1.5–2.0 cm wide. Mature *S. alba* fruit were characterized by a size of 40 mm or more, and mature fruits contained 150–200 seeds.

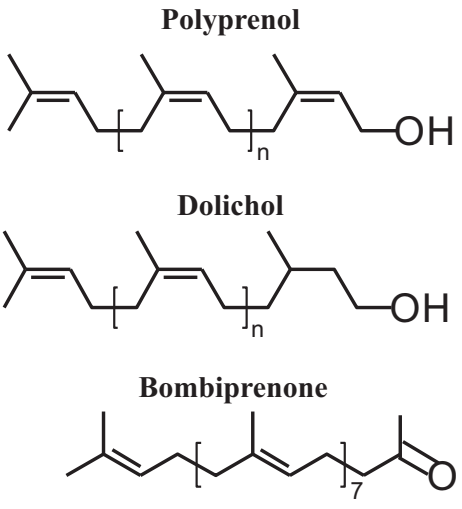

Figure 1. Structure of polyprenol, dolichol, and bombiprenone. n shows the number of internal isoprene residues.

The germinated seedlings grew for 5 months under exposure to natural temperature and sunlight in an uncontrolled glass house. The maximum irradiance in the glass house was 950 μmol m^{-2} s^{-1}, and the average temperature was 24.1°C. A seawater solution was prepared by dissolving commercial salt powder (Red Sea Salt, Houston, TX, USA) to make 0% and 3% (equal to seawater level) salinity concentrations in accordance with the manufacturer's protocol. Salinity in this study was measured as the mass of salt powder/weight of solution (Basyuni *et al.* 2012b). The salt concentration in each pot treatment in this study was checked weekly during the experiments by an S/Mill-E Salinity Refractometer (ATAGO Co., Ltd., Tokyo, Japan) and was adjusted by adding tap water for the control (0%) or pure water (salt treatments) to compensate for water lost because of evapotranspiration. Three plants in a separate pot, that is, five portable pots per species per salinity treatment, were grown for 5 months. After 5 months of cultivation, the four species of mangrove plants were harvested and washed, after which the leaves and roots were flash frozen in liquid nitrogen and then stored at −20°C for further analysis.

2.3. Isolation of polyisoprenoid alcohols

The procedure for the isolation of polyisoprenoids was performed, as previously described (Basyuni *et al.* 2017a). The leaves and roots of *A. marina*, *B. gymnorrhiza*, *K. obovata*, and *S. alba* seedlings under 0% and 3% salt concentrations were dried at 75°C for 1–2 days. The dried tissue (2–4 g each) was crushed to a fine powder, and then immersed in 30 mL of chloroform/methanol (2:1, v/v) solvent for 48 h. The lipid extract of the leaves and roots was saponified at 65°C for 24 h in 50% ethanol containing 2 M KOH. The NSLs of each tissue were extracted with hexane, and the organic solvent was evaporated and redissolved in hexane. The leaf (≈100 μg) and root (≈150 μg) extracts were added to each TLC plate.

2.4. Investigation of polyisoprenoids by two-dimensional thin layer chromatography

First-dimensional TLC was carried out for approximately 60 min on a silica gel glass plate (20 × 3 cm) with a solvent system of toluene-ethyl acetate (9:1), as previously described (Basyuni *et al.* 2016; 2017a). Two-dimensional reversed-phase C-18 silica gel HPTLC was performed with acetone as the solvent for approximately 40 min. The position of the separated polyisoprenoid alcohols by two-dimensional silica gel TLC was identified and visualized with iodine vapor. To determine whether the family corresponded to dolichols or polyprenols, dolichol or polyprenol reference standards were added to the sample line of the first-dimension TLC and developed with a solvent system, as previously described (Basyuni *et al.* 2016). The developed chromatographic images were obtained and digitally scanned with a PIXMA G2000 Canon Series Printer (Canon Singapore Pte. Ltd.). The polyisoprenoid family was identified via the comparison of mobility in TLC with that of authentic

standards of dolichol or polyprenol applied during the two-dimensional development. The polyprenols and dolichols that were detected on the RP-18 HPTLC glass plates were quantified using ImageJ 1.46r software (NIH, Bethesda, USA) (Schneider *et al.* 2012), and dolichol and polyprenol standards served as references.

3. Results

We analyzed the effects of salinity (0% and 3%) on the occurrence, content, and distribution of polyisoprenoids in four mangrove seedlings—two secretors (*A. marina* and *S. alba*) and two non–salt-secretors (*B. gymnorrhiza* and *K. obovata*)—by the two-dimensional thin layer chromatography method (Basyuni *et al.* 2016, 2017a). Separation of polyisoprenoids into polyprenols and dolichols with different chain lengths was observed. The structural groups of the polyprenols and dolichols in the leaves were classified into two types (I and II), as previously described (Basyuni *et al.* 2016, 2017a). In type I, which exhibits a predominance of dolichols over polyprenols (more than 90%), and in type II, the occurrence of both polyprenols and dolichols was detected. Type III groups, which display a predominance of polyprenols over dolichols, were not observed in this study.

Table 1 lists the polyisoprenoid concentrations and distributions in the leaves of the four species grown for 5 months under 0% and 3% salinity levels. Salinity altered the content of polyisoprenoids and dolichols in the leaves of *A. marina*, *B. gymnorrhiza*, and *K. obovata* (Table 1). A similar change was also found for the relative proportion of polyisoprenoids and dolichols in the total NSLs in the leaves of *A. marina*, *B. gymnorrhiza*, and *K. obovata*. It is interesting to note that polyprenols were not detected in the 0% (control) group of *A. marina* leaves and that small amounts were observed in the *B. gymnorrhiza* and *K. obovata* leaves; however, significant amounts were found in the 3% salinity group. These findings regarding the *A. marina*, *B. gymnorrhiza*, and *K. obovata* leaves imply changes in the structural group: under 0% salt concentrations, these groups are classified as type I, but the groups become type II at the 3% salinity level.

In contrast, we observed an approximately 0.7-fold decrease in the concentration of polyisoprenoids and polyprenols in the *S. alba* leaves in the 3% salt treatment. Only dolichols increased with increasing salt concentration in the *S. alba* leaves. A similar result for the structural group was also shown in the *S. alba* leaves: both the salt-treated and control groups consisted of type II. Dolichols predominated over polyprenols in the mangrove leaves of the control treatment; however, in the 3% salt group, the presence of polyprenols and dolichols were occurred (Table 1).

Table 1 displays that salt concentration significantly increased the bombiprenone content in the leaves of *B. gymnorrhiza* and *S. alba*. In contrast to this observation, no change of bombiprenone content in *K. obovata* leaves. It is noteworthy that bombiprenone content was not detected in *A. marina* leaves under 0% salinity, and found abundantly in 3% salinity.

Table 2 summarizes the occurrence and distribution of polyisoprenoid alcohol content in the roots of *A. marina*, *B. gymnorrhiza*, *K. obovata*, and *S. alba* grown for 5 months under 0% and 3% salinity concentrations. Salt stress increased the concentrations of polyisoprenoids, polyprenols, and dolichols in the roots of *A. marina*, *K. obovata*, and *S. alba*, but decreased the polyisoprenoids and dolichols in *B. gymnorrhiza* roots, as shown in Table 2.

A similar increase was also found in the relative proportion of polyisoprenoids, polyprenols, and dolichols in the total NSLs in the roots of *A. marina*, *B. gymnorrhiza*, *K. obovata,* and *S. alba* in the 3% salt group. It is noteworthy that the polyisoprenoid type in the roots of the two salt-secreting species *A. marina* and *S. alba* consists of type I under both the 0% and 3% salt concentrations. On the other

Table 1. Effect of salinity on the occurrence and distribution of polyisoprenoids (Bom, FP, Pol, and Dol) in four mangrove leaves

Species	Salinity (‰)	PI (mg/g dw)	Bom (mg/g)	FP (mg/g)	Pol (mg/g)	Dol (mg/g)	% in total NSL				% in polyisoprenoid				Type
							PI	Bom	FP	Pol	Dol	Bom	FP	Pol	Dol	
A. marina	0.0	18.6 ± 0.4	nd	nd	nd	18.6 ± 0.4	26.2 ± 5.4	nd	nd	nd	26.2 ± 5.4	nd	nd	nd	100.0 ± 0.0	I
B. gymnorrhiza	0.0	22.2 ± 9.1	9.8 ± 4.4	nd	3.1 ± 0.1	9.3 ± 4.7	13.8 ± 6.6	5.5 ± 1.8	nd	2.8 ± 1.1	5.5 ± 3.3	5.1 ± 3.6	nd	3.7 ± 2.4	91.2 ± 1.6	I
K. obovata	0.0	54.3 ± 4.0	23.4 ± 22.2	nd	2.1 ± 0.1	28.8 ± 3.8	73.1 ± 73.5	31.8 ± 4.1	nd	2.7 ± 1.7	38.6 ± 37.6	4.5 ± 5.9	nd	5.2 ± 2.7	90.3 ± 3.1	I
S. alba	0.0	454.9 ± 15.3	0.1 ± 0.1	nd	310.5 ± 14.2	144.3 ± 7.4	337.5 ± 10.1	0.0 ± 0.0	nd	231.7 ± 11.0	105.8 ± 8.2	0.8 ± 0.0	nd	59.9 ± 13.8	39.3 ± 2.4	II
A. marina	3.0	131.6 ± 16.2*a*	52.9 ± 7.8	33.6 ± 5.2	2.8 ± 0.2	42.3 ± 2.3*a*	317.8 ± 6.5*a*	49.0 ± 7.7	39.7 ± 5.6	59.6 ± 3.6	169.5 ± 36.1*a*	20.7 ± 1.7	10.1 ± 1.0	16.0 ± 22.7	53.2 ± 6.9*a*	II
B. gymnorrhiza	3.0	63.6 ± 1.4*a*	36.8 ± 5.3*a*	8.4 ± 1.8	8.9 ± 2.4	9.5 ± 1.9*a*	14.2 ± 7.1	3.2 ± 1.8	3.6 ± 1.0	3.5 ± 2.1	3.9 ± 2.0	22.7 ± 1.5*a*	26.7 ± 6.3	24.0 ± 3.2*a*	26.6 ± 1.6*a*	II
K. obovata	3.0	109.0 ± 14.8*a*	20.8 ± 6.0	33.0 ± 4.1	25.8 ± 1.5*a*	29.4 ± 2.6	87.0 ± 7.1	15.5 ± 1.2*a*	26.8 ± 6.6	19.7 ± 4.9*a*	25.0 ± 17.4	19.6 ± 4.2*a*	27.4 ± 8.1	23.5 ± 2.1*a*	29.5 ± 1.7*a*	II
S. alba	3.0	349.0 ± 4.7*a*	64.9 ± 4.2*a*	nd	138.3 ± 9.0*a*	145.8 ± 7.3*a*	300.2 ± 9.3	53.5 ± 4.9*a*	nd	120.5 ± 4.2*a*	126.2 ± 4.0*a*	18.6 ± 6.5*a*	nd	39.5 ± 4.2*a*	41.9 ± 2.3	II

Data are represented as the means ± standard error ($n = 3$) at least three independent experiments. *a*Significantly different from 0‰ at $P < 0.05$ using Dunnett's test.
Bom = bombiprenone; Dol = dolichols; FP = ficaprenol; nd = not detected; NSL = nonsaponifiable lipid; PI = polyisoprenoids; Pol = polyprenols

Table 2. Effect of salinity on the occurrence and distribution of polyisoprenoids in four mangrove roots

Species	Salinity (%)	PI (mg/g dw)	Bom (mg/g)	FP (mg/g)	Pol (mg/g)	Dol (mg/g)	% in total NSL					% in polyisoprenoid				Type
							PI	Bom	FP	Pol	Dol	Bom	FP	Pol	Dol	
A. marina	0.0	15.6 ± 2.3*a*	nd	nd	nd	15.6 ± 2.3*a*	29.3 ± 1.8	nd	nd	nd	29.3 ± 1.8	nd	nd	nd	100 ± 0.0	I
B. gymnorrhiza	0.0	40.7 ± 5.1*a*	nd	nd	7.0 ± 1.6*a*	33.7 ± 4.0*a*	58.5 ± 3.1	nd	nd	9.8 ± 1.1	48.6 ± 4.2	nd	nd	17.1 ± 2.7	82.9 ± 2.7	II
K. obovata	0.0	40.5 ± 6.0*a*	nd	nd	13.2 ± 4.8*a*	27.3 ± 2.1*a*	63.4 ± 4.2	nd	nd	20.2 ± 2.8	43.3 ± 3.9	nd	nd	34.0 ± 1.6	66.0 ± 1.6	II
S. alba	0.0	27.1 ± 1.7*a*	nd	nd	2.7 ± 0.4*a*	24.4 ± 1.6*a*	27.8 ± 3.2	nd	nd	2.8 ± 0.7	25.0 ± 2.6	nd	nd	10.0 ± 1.3	90.0 ± 1.2	I
A. marina	3.0	25.2 ± 1.7*a*	nd	nd	nd	25.2 ± 1.7*a*	78.0 ± 5.5	nd	nd	nd	78.0 ± 5.5*a*	nd	nd	nd	100.0 ± 0.0	I
B. gymnorrhiza	3.0	30.8 ± 2.6*a*	nd	nd	8.0 ± 3.5*a*	22.7 ± 3.1*a*	69.1 ± 4.0*a*	nd	nd	18.0 ± 2.8*a*	51.1 ± 4.1	nd	nd	25.7 ± 1.7*a*	74.3 ± 1.7*a*	II
K. obovata	3.0	76.1 ± 1.1*a*	nd	nd	22.5 ± 1.4*a*	53.6 ± 0.9*a*	87.5 ± 8.0*a*	nd	nd	33.6 ± 8.1*a*	53.9 ± 0.8*a*	nd	nd	29.4 ± 1.4	70.6 ± 0.6	II
S. alba	3.0	44.9 ± 3.0*a*	nd	nd	2.9 ± 0.5*a*	42.0 ± 3.8*a*	55.9 ± 4.9*a*	nd	nd	4.1 ± 0.7	51.8 ± 5.6*a*	nd	nd	7.7 ± 1.8	92.3 ± 2.0	I

Data are represented as the means ± standard error ($n = 3$). [a]Significantly different from 0% at $P < 0.05$ using Dunnett's test.
Bom = bombiprenone; Dol = dolichols; FP = ficaprenol; nd = not detected; NSL = nonsaponifiable lipid; PI = polyisoprenoids; Pol = polyprenols.

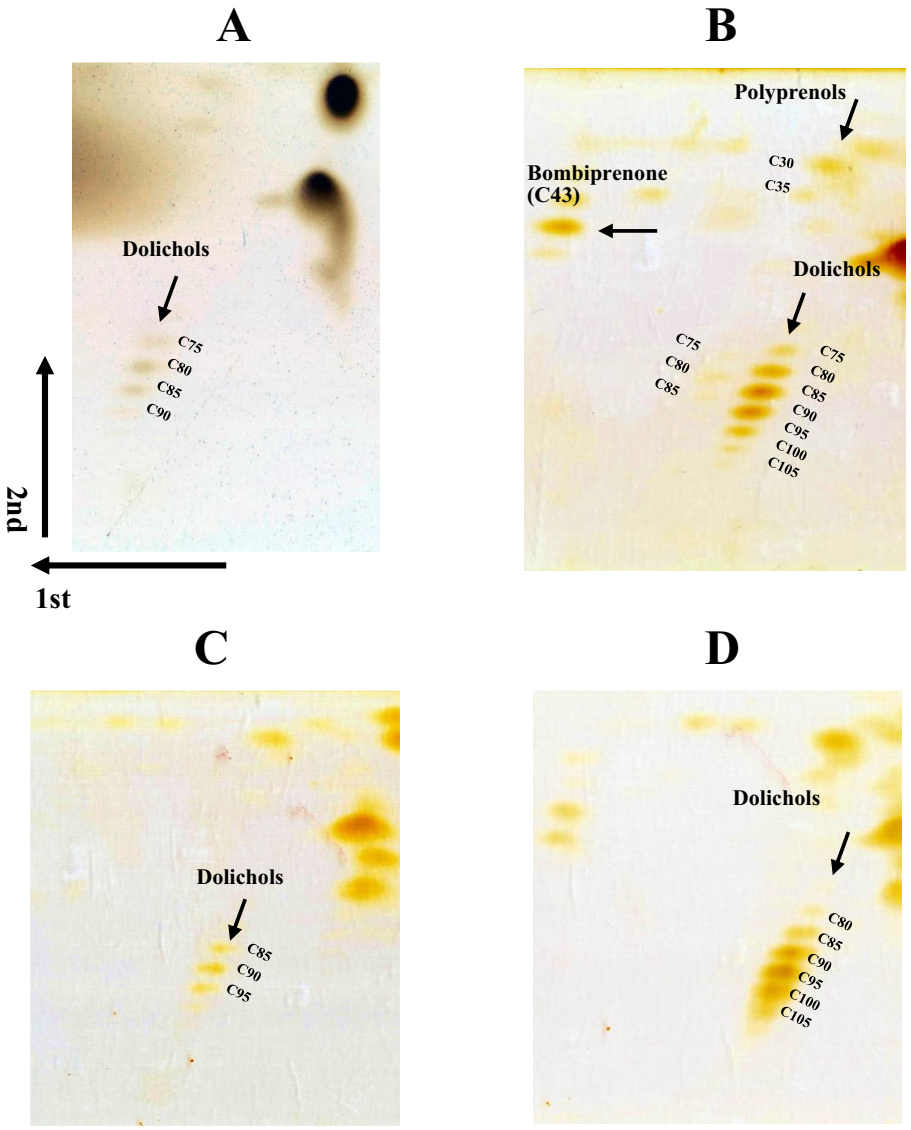

Figure 2. 2D-TLC chromatograms of polyisoprenoids from *A. marina* leaves in 0% salinity (A) and 3% salinity (B) , and *A. marina* roots in 0% salinity (C) and 3% salinity (D). Data are the mean of three independent experiments. The number showing the carbon chain length of polyisoprenoid alcohols. Arrow shows the direction of TLC phase. 2D-TLC = two-dimensional thin layer chromatography.

hand, type II was observed in the non–salt-secreting species *B. gymnorrhiza* and *K. obovata*.

Figure 2 shows the two-plate TLC chromatograms of the polyisoprenoids of *A. marina* leaves and roots under the 0% and 3% salinity treatments. Dolichols with chain lengths of C_{75}–C_{90} were detected as the primary polyisoprenoid alcohols in *A. marina* leaves under 0% salt concentration (Figure 2A). In contrast, polyprenols with chain lengths of C_{75}–C_{85} were detected in trace amounts in *A. marina* leaves under 3% salt concentrations (Figure 2B), as were longer-chain dolichols—C_{75}–C_{105} (Figure 2B; Table 3).

Table 3. Carbon chain lengths of bombiprenone, polyprenol, and dolichol occurring in four mangrove seedlings under 0% and 3% salt concentration

Species	Tissue	0 % salinity			3% salinity		
		Bom	Pol	Dol	Bom	Pol	Dol
A. marina	leaves	nd	nd	75 80 85 90	○	75 80 85	75 80 85 90 95 100 105
B. gymnorrhiza	leaves	○ 85 90		75 80 85 90 95 100	○ 50 55	85 90	75 80 85 90 95 100
K. obovata	leaves	○ 80 85		75 80 85 90	○ 45 50 55	80 85 90	75 80 85 90 95
S. alba	leaves	○ 65 70 75 80 85 90 95 100 105 110 115 120 125 130 135 140		65 70 75 80 85 90 95 100 105 110 115 120 125 130	○	90 95 100 105 110	65 70 75 80 85 90 95 100 105 110
A. marina	roots		nd	85 90 95		nd	80 85 90 95 100 105
B. gymnorrhiza	roots	70 75 80		70 75 80 85 90 95		70 75 80	70 75 80 85 90 95
K. obovata	roots	85 90 95 100		80 85 90 95 100		85 90 95 100	80 85 90 95 100 105
S. alba	roots	85 90 95		75 80 85 90 95 100 105 110		85 90 95	75 80 85 90 95 100 105 110 115 120

The numbers refer to the carbon-chain length of polyisoprenoid alcohols. Bom = Bombiprenone, Pol = Polyprenols, Dol = Dolichols

Bombiprenone (C_{43}) was detected in *A. marina* leaves under 3% salt (Figure 2B).

In the cases of *A. marina* roots under 0% and 3% salt concentrations and *A. marina* leaves under 0% salinity, no polyprenols were detected. Dolichols with different chain lengths were detected in *A. marina* roots under 0% salt (C_{85}–C_{95}) and 3% salinity (C_{80}–C_{105}). It is interesting to note that dolichols but not polyprenols had much longer chain lengths in both *A. marina* leaves and roots under salinity conditions (Table 2; Figures 2B and D).

Figure 3 shows the two-dimensional TLC chromatogram of polyisoprenoids from the leaves and roots of *B. gymnorrhiza* under 0% and 3% salt concentrations. Dolichols consisting of C_{75}–C_{100} were the major polyisoprenoid alcohols in *B. gymnorrhiza* leaves under 0% and 3% salt concentrations (Figures 3A and 3B). The 3% salt group was distinguishable from the 0% group by the occurrence of ficaprenols (C_{50}–C_{55}), as shown in Figure 3B. In dolichol-rich tissue, longer polyprenols (C_{85}–C_{90}) were also found in *B. gymnorrhiza* leaves (Table 3; Figure 3A and B). Polyprenols with chain lengths of C_{70}–C_{80} and dolichols with chain lengths of C_{70}–C_{80} were detected in the roots of *B. gymnorrhiza* (Table 3; Figures 3C and 3D) in both the 0% and 3% salt groups. Bombiprenone (C_{43}) was found in the leaves of *B. gymnorrhiza* in both the control- and salt-treated groups (Figures 3A and 3B).

Figure 4 depicts two-plate TLC chromatograms of polyisoprenoids from the leaves and roots of *K. obovata* under 0% and 3% salinity concentrations. As similar result to *B. gymnorrhiza* leaves in 3% salt group, the presence of ficaprenols (C_{45}–C_{55}) was also found in *K. obovata* under 3% salt concentrations, as shown in Figure 4B.

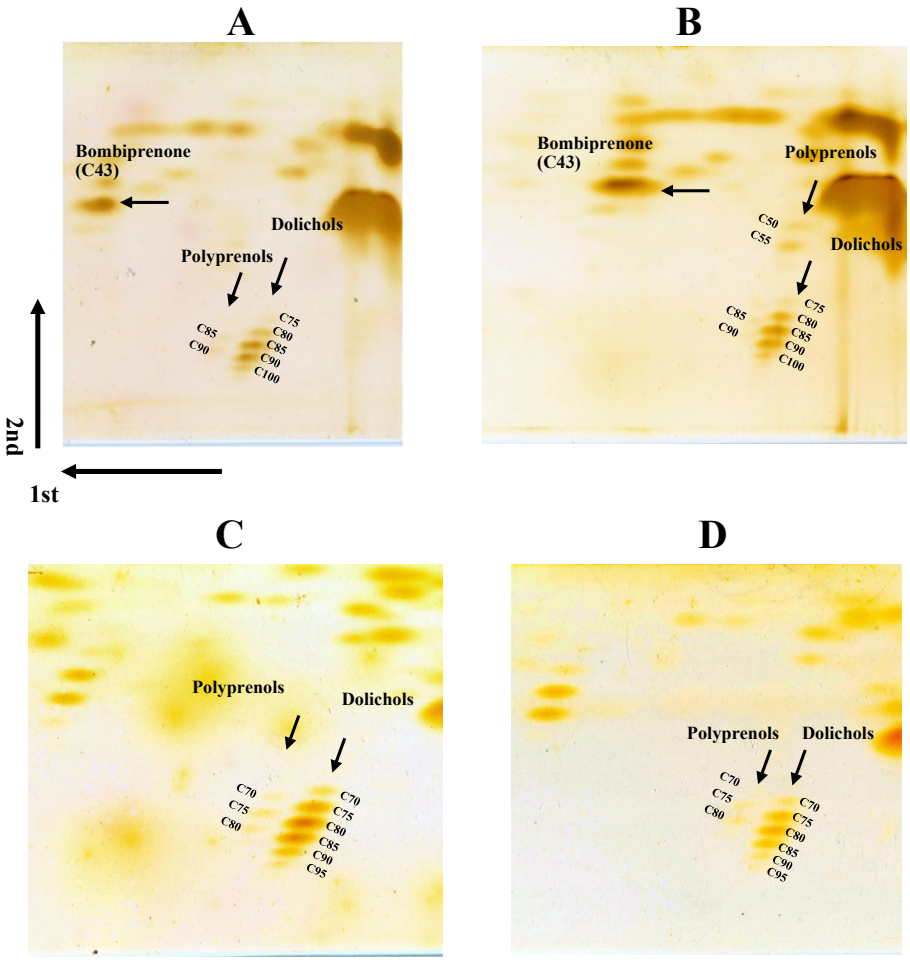

Figure 3. 2D-TLC chromatograms of polyisoprenoids from *B. gymnorrhiza* leaves in 0% salinity (A) and 3% salinity (B), and *B. gymnorrhiza* roots in 0% salinity (C) and 3% salinity (D). Data are the mean of three independent experiments. The number showing the carbon chain length of polyisoprenoid alcohols. Arrow shows the direction of TLC phase. 2D-TLC = two-dimensional thin layer chromatography.

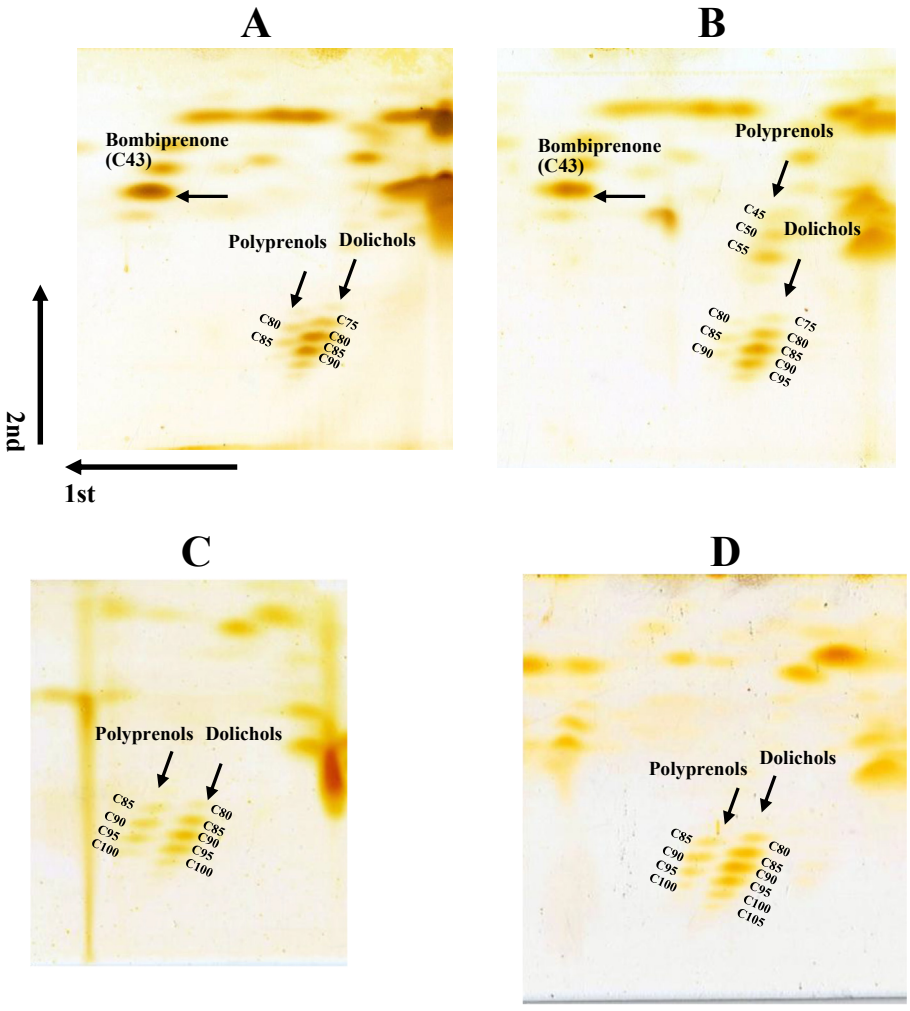

Figure 4. 2D-TLC chromatograms of polyisoprenoids from *K. obovata* leaves in 0% salinity (A) and 3% salinity (B), and *K. obovata* roots in 0% salinity (C) and 3% salinity (D). Data are the mean of three independent experiments. The number showing the carbon chain length of polyisoprenoid alcohols. Arrow shows the direction of TLC phase. 2D-TLC = two-dimensional thin layer chromatography.

The longer polyprenols and dolichols (>C_{75}) occurred in the leaves of *K. obovata* under 0% and 3% salinity group. Polyprenols with chain lengths of C_{85}–C_{100} and dolichols with chain lengths of C_{80}–C_{100} were detected in the roots of *K. obovata* (Table 3; Figures 4C and 4D) in both the 0% and 3% salt groups. In case of *K. obovata* roots under 3% salt concentration, dolichol with chain length of C_{105} was detected. Bombiprenone (C_{43}) was detected in the leaves of *K. obovata* in both the control- and salt-treated groups (Figures 4A and 4B).

Figure 5 also shows the two-dimensional TLC chromatogram of polyisoprenoids from the leaves and roots of *S. alba* under 0% and 3% salt concentrations. Polyprenols with C_{65}–C_{140} and dolichols with C_{65}–C_{130} were detected in the leaves of *S. alba* under 0% salinity (Figure 5A). In contrast, the chain lengths of polyprenols and dolichols in *S. alba* leaves under 3% salt concentration were much shorter than that in 0% salinity (Figure 5B). Polyprenols with chain lengths of C_{85}–C_{95} were detected in *S. alba* roots in 0% and 3% salt group (Figures 5C and 5D). Bombiprenone was detected only in *S. alba* leaves under 0% and 3% salt group.

4. Discussion

The present study describes the polyisoprenoid concentrations in four seedlings of mangrove species in response to salt stress. Understanding the salt tolerance mechanism of mangrove plants requires a comprehensive observation of stress-induced changes in polyisoprenoid alcohol content. Our previous studies on the polyisoprenoid occurrence and contents in Okinawan and Indonesian mangroves (Basyuni *et al.* 2016, 2017a) enabled us to study the salt-dependent changes in polyisoprenoid concentrations in mangrove seedlings for the first time. The observations of salt-dependent changes in polyisoprenoid concentrations in the present study thus can shed some light on the mechanisms of plant adaptations to the salt stress.

The physiological significance of polyisoprenoids in leaves appeared to differ between the salt-secreting and the non–salt-secreting mangrove species studied. The presence of ficaprenols (C_{50}–C_{55}) or (C_{45}–C_{55}) observed in the leaves of the non–salt-secretors *B. gymnorrhiza* and *K. obovata* under 3% salinity was not observed in the leaves of any secreting species under 3% salt concentration. This finding may be explained by the slow pyrophosphorylation of short-chain prenols, such as in ficaprenol (Rip *et al.* 1985). Different stereochemistry between short- and long-chain polyprenols and the control of the chain length specificity of these compounds remain obscure (Swiezewska and Danikiewicz 2005). Recently, it was shown that both short- and long-chain polyisoprenoid alcohols exhibit different levels of thermooxidation (Molińska *et al.* 2015). This result suggested that leaves are

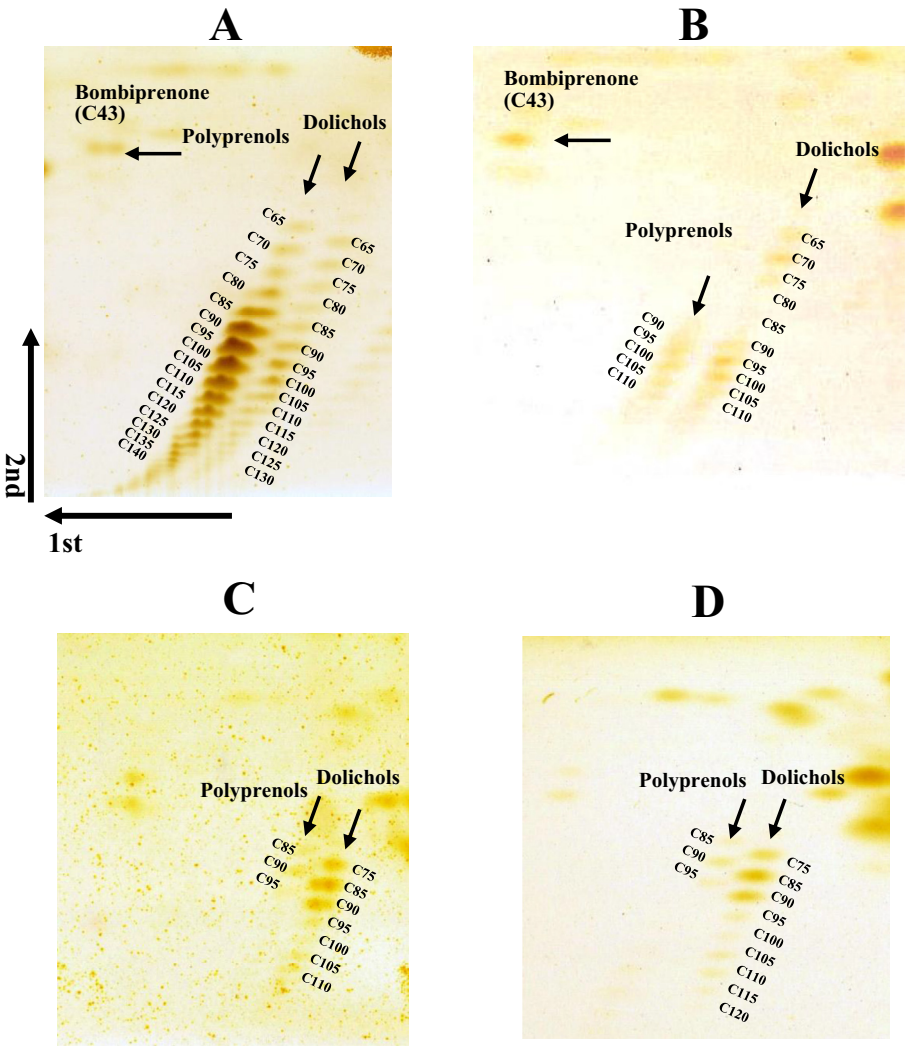

Figure 5. 2D-TLC chromatograms of polyisoprenoids from *S. alba* leaves in 0% salinity (A) and 3% salinity (B), and *S. alba* roots in 0% salinity (C) and 3% salinity (D). Data are the mean of three independent experiments. The number showing the carbon chain length of polyisoprenoid alcohols. Arrow shows the direction of TLC phase. 2D-TLC = two-dimensional thin layer chromatography.

capable of synthesizing various arrays of secondary metabolites for self-defense.

The salt tolerance mechanism of mangroves can be divided into three types: salt excluders or non–salt-secretors, salt accumulators, and salt secretors (Basyuni *et al.* 2011, 2012a; Parida and Jha 2010; Tomlinson 1986). Each mangrove species has mechanisms to regulate the levels of salt in their sap. Plants that exclude salt prevent the sap from entering the membranes of their roots during water uptake (Basyuni *et al.* 2012a). They can selectively take up only positive ions (electrically charged atom(s) and the group of the atom(s) whose salts are in solution) from the solutions they come into contact with by a process called ultrafiltration (Parida and Jha 2010). This mechanism occurred in the non–salt-secretors *B. gymnorrhiza* and *K. candel.* Other mangrove plants mature while containing excess salt; some accumulate the excess salt into older leaves, whereas other mangrove plants excrete salt in higher concentrations than seawater through salt hairs on their leaves (Parida and Jha 2010; Tomlinson 1986).

The different salt mechanisms of mangroves lead to different chain lengths of polyisoprenoids—from shorter to longer ones (Basyuni *et al.* 2016). Accumulations of ficaprenol have been identified in the yellow leaves of *K. obovata* (Basyuni *et al.* 2016), this species accumulates salt in its older leaves. These results are in agreement with those of previous reports regarding the accumulation of short-chain polyprenols (C_{45}–C_{60}) of *Tilia* "Euchlora" leaves under conditions of increased soil salinity (Baczewska *et al.* 2014). Shorter chains of polyprenols have been characterized in the families of Euphorbiaceae (Swiezewska *et al.* 1994), Lauraceae, Tiliaceae, and Magnoliaceae (Roslinska *et al.* 2002); in soybean leaves (Kurisaki *et al.* 1997); in young and old rubber leaves (Tateyama *et al.* 1999); in spinach leaves (Sakaihara *et al.* 2000); and in the leaves of several mangrove species such as *Excoecaria agallocha*, *Heritiera littoralis*, *Hibiscus tiliaceus* (Basyuni *et al.* 2016), *Acrostichum aureum*, *Avicennia officinalis*, and *Rhizophora apiculata* (Basyuni *et al.* 2017a). The occurrence of ficaprenols in *B. gymnorrhiza* and *K. obovata* leaves and in other abovementioned plants suggested the localization of ficaprenols in the chloroplasts (Kurisaki *et al.* 1997; Sakaihara *et al.* 2000). The presence of phytol, probably derived from chlorophyll, was also detected in *B. gymnorrhiza* and *K. obovata* leaves (Basyuni *et al.* 2007). For reasons unknown, accumulated ficaprenols were detected only in *B. gymnorrhiza* and *K. obovata* leaves under 3% salinity.

Polyisoprenoids, similar to previous reports on triterpenoids (Basyuni *et al.* 2009, 2012b), modulate salt tolerance with respect to the polyprenyl diphosphate synthase gene as well as the physicochemical properties of cell membranes by increasing the permeability and adaptation to external stress (Basyuni and Wati 2017b; Inafuku *et al.* 2016). Dolichol contents in the roots of *Coluria geoides* and *Cucumis sativus* increased under all abiotic stress conditions, including salinity, heavy metal, and low temperatures (Skorupinska *et al.* 2009). Similarly, dolichol increases the drought resistance of *A. thaliana* (Zhang *et al.* 2008). These studies support our present research regarding the accumulation of dolichols in three mangrove seedlings. Polyprenols are the dominant polyisoprenoids in the plant world (especially in photosynthetic tissues); however, in mangroves, dolichols predominated over polyprenols in the leaves (Basyuni *et al.* 2016, 2017a). This finding adds to previous reports on the sources of dolichols, which include plant roots, yeast, and animal tissue (Basyuni *et al.* 2016, 2017a; Surmacz and Swiezewska 2011; Swiezewska and Danikiewicz 2005; Swiezewska *et al.* 1994). The occurrence of multiple families of polyisoprenoids in plant tissues, including in the present study, might be a product of different biosynthetic pathways in plants (Swiezewska and Danikiewicz 2005).

The predominance of dolichols over polyprenols in some plant tissues in conjunction with polyprenols dominating over dolichols in this study and in other reports is still an unresolved topic. It has been suggested that this phenomenon might result from the alternating balance of specific compounds of biosynthetic end-products (Skorupinska *et al.* 2007). Furthermore, the predominance of dolichols over polyprenols in mangrove leaves may be due to the presence of active polyprenol reductase enzymes (Basyuni *et al.* 2016, 2017a) as a consequence of tropical or subtropical conditions.

Plant cells can produce a diverse array of secondary metabolites for self-defense (Jwa *et al.* 2006; Khan *et al.* 2017). The isoprenoid family has been shown to be biologically active during the protection of plants from external attacks, including attacks by insect herbivores as well as bacterial and viral infections (Jwa *et al.* 2006; Osboum *et al.* 2003). Our previous study suggested that terpenoids play a role in the protection of mangrove plants from salt stress (Basyuni *et al.* 2009, 2012a). Furthermore, a substantial amount of triterpenoids distributed in the outer parts of the root combined with the data from the present study may provide strong evidence for the protective roles of triterpenoids in mangrove species (Basyuni *et al.* 2007). Triterpenoids from *Rhizophora mangle* may function as chemical defense substances, as they show insecticidal activity (Williams 1999). In this respect, polyisoprenoids have been suggested to play a major role in defense mechanisms against adverse environmental stresses (Molińska *et al.* 2015).

In conclusion, this study confirms that the physiological significance of polyisoprenoids in non−salt-secreting species may differ from that of salt-secreting species. Therefore, it is likely that polyisoprenoid plays a protective role in mangrove leaves from salinity in both salt secretors and non−salt-secretors. Additional studies are necessary to get more insight into the role of polyisoprenoid alcohol content against environmental stresses and to better understand the expression of polyisoprenoid genes in mangrove plants.

Conflict of Interest Statement

The authors declare that there is no conflict of interest.

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